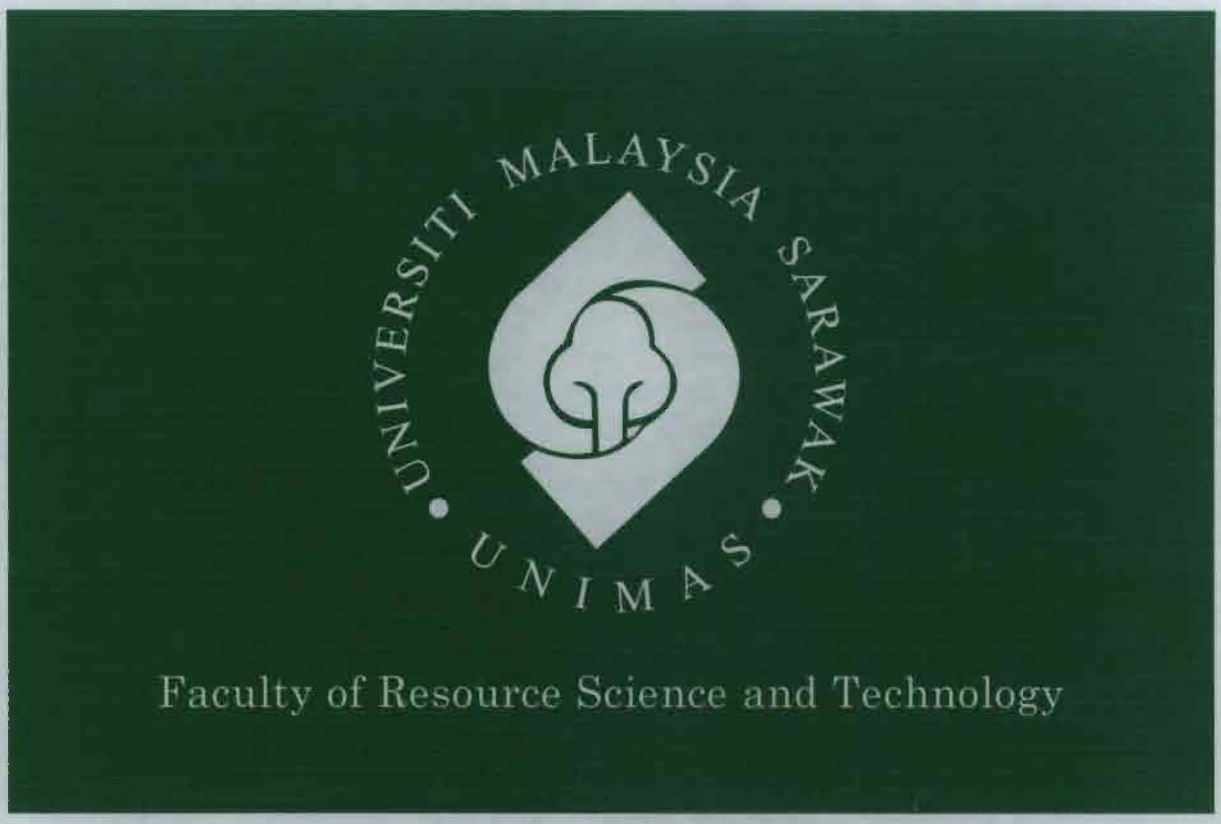

THE INFLUENCE OF STEAMING PRETREATMENT ON SUGARS RECOVERY UPON COMPLETING HYDROLYSIS PROCESS FROM RESIDUAL STARCH OF SAGO HAMPAS

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Declaration

"I hereby declare that this dissertation entitled **'The Influence of Steaming Pretreatment on Sugars Recovery upon Completing Hydrolysis Process from Residual Starch of Sago Hampas'** is based on my original work and research, except for quotation and citation, which has been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or any other institution."

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rpm	Revolution per minute
KH_2PO_4	Potassium dihydrogen phosphate
HPLC	High Performance Liquid Chromatography
SEM	Scanning Electron Microscope

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ABSTRACT

Currently, there is a wide utilisation of agricultural waste especially sago hampas where the hampas which contains starch and lignocellulose, has shown its capability to be converted into sugar through enzymatic and acid hydrolysis. These sugars can be further fermented and converted into value added products such as bioethanol. However, residual starch in sago hampas are trapped in the lignocellulose matrix and natural lignocellulose does not undergo enzymatic hydrolysis efficiently due to the nature of the lignocellulosic structure. Steaming pretreatment of lignocellulosic compound alters the construction of cellulosic biomass which causes the starch and cellulose in the plant fibres to be exposed and be more accessible. The focus of this study is the recovery of sugar from residual starch of sago hampas through steaming pretreament and enzymatic hydrolysis of starch. 7% substrate load of sago hampas at pH 4 shows the highest hydrolysis yield of 69.04% at only 30 min while 10% substrate load shows the highest yield at pH 5 and 30 min with hydrolysis yield of 65.49%. Significant difference in glucose concentration can be observed when compared to the controls of the experiment thus steaming pretreatment influences the sugars recovery of residual starch of sago hampas.

Keywords: Steaming pretreatment, sago hampas, enzymatic hydrolysis, residual starch

ABSTRAK

Pada masa ini, terdapat penggunaan daripada sisa pertanian seperti hampas sagu kerana ia mengandungi kanji dan lignosellulosa, dan telah menunjukkan keupayaannya untuk ditukar menjadi gula melalui enzim dan asid hidrolisis. Gula ini boleh terus difermentasi dan ditukar menjadi produk tambah-nilai seperti bioetanol. Walau bagaimanapun, sisa kanji di dalam hampas sagu terperangkap pada matrik lignosellulosa dan lignosellulosa semula jadi tidak menjalani hidrolisis enzim berkesan kerana sifat strukturnya. Prarawatan mengukus kompaun lignoselulosa mengubah pembinaan biomas sellulosa yang menyebabkan kanji dan selulosa di dalam serat tumbuh-tumbuhan akan terdedah dan menjadi lebih mudah dicapai. Fokus dalam penyelidikan ini adalah mendapatkan gula daripada sisa kanji pada hampas sagu melalui prarawatan mengukus dan hidrolisis enzim pada kanji. Hampas sagu dengan paras substrat 7% pada pH 4 menunjukkan hasil hidrolisis tertinggi dengan 69.04% pada hanya 30 minit manakala paras substrat 10% menunjukkan hasil hidrolisis tertinggi pada pH 5 dan 30 minit dengan hasil hidrolisis 65.49%. Perbezaan yang signifikan pada kepekatan glukosa dapat diperhatikan apabila dibandingkan dengan kawalan eksperimen dengan itu prarawatan mengukus mempengaruhi pemulihan gula dari sisa kanji hampas sagu.

Kata kunci: prarawatan mengukus, hampas sagu, hidrolisis enzim, sisa kanji

1.0 Introduction

Sago hampas is an example of an agricultural waste that has been widely utilised as animal feedstock, compost for culture of mushroom, for particleboard manufacture and for hydrolysis to confectioners' syrup (Singhal et al., 2008). Apart from that, the hampas which contains residual starch and lignocellulose, has also shown its capability to be converted into sugar through enzymatic (Awg-Adeni et al., 2010). These sugars can be further fermented and converted into value added products such as bioethanol.

The problems associated with residual starch in sago hampas that are trapped in the lignocellulose matrix and natural lignocellulose that are directly hydrolysed by enzymes are the inefficient enzymatic hydrolysis which leads to low sugar yield. This is due to the nature of the lignocellulosic structure in the sago hampas. This low yield directly affects the cost of production where it is not cost effective as high amount of substrate is used but low amount of sugar is produced. Therefore, the raw material necessitates some method of pretreatment to expose the structure of lignocellulosics as well as starch to ensure efficient enzymatic is accomplished.

The beneficial effects of pretreatment of lignocellulosic materials have been recognized for a long time. The goal of the pretreatment process is to release the starch granules trapped in the lignocellulosic matrix, remove lignin and hemicellulose, reduce the crystallinity of cellulose, and increase the porosity of the lignocellulosic materials (Kumar et al., 2009). Pretreatment must meet the following requirements: (1) improve the formation of sugars or the ability to subsequently form sugars by hydrolysis, (2) avoid the degradation or loss of

carbohydrate, (3) avoid the formation of by-products that are inhibitory to the subsequent hydrolysis and fermentation processes, and (4) be cost-effective (Kumar et al., 2009).

The focus in this study is the enzymatic hydrolysis of starch upon completing steaming pretreatment. The methodology used in this experiment includes liquefaction and saccharification of sago hampas where Liquozyme SC DS enzyme was used for liquefaction while Spirizyme Fuel was used for saccharification process. The parameters involved in the experiment that were manipulated to determine the optimum condition was the substrate load which is varied at 7% and 10% as well as the pH value during enzymatic hydrolysis (pH 4 and 5) which were used to suspend the sago hampas.

The objectives for this experiment includes:

1. To determine the optimum parameters for steaming pretreatment of sago hampas
2. To analyse the sugar production upon completing steaming pretreatment and enzymatic hydrolysis of starch
3. To observe the changes in the microstructure of sago hampas before and after the steaming pretreatment

2.0 Literature Review

2.1 Sago Hampas

Sago 'hampas' is an inexpensive, starchy lignocellulosic, copious fibrous residue left over after most of the starch in the rasped pith of the *Metroxylon sagu* (sago palm) has been washed out (Singhal et al., 2008). The amount of 'hampas' released from the sago processing factory hinge on mostly on the excellence of the extraction procedure. Sago hampas contains 23% cellulose, 9.2% hemicellulose, 4% lignin and 58% starch on a dry weight basis (Awg-Adeni et al., 2013). Since this contingent mostly on the excellence of the extraction procedure, it has been found that dried fibrous sago waste comprise about 60-70% dry weight of starch (Kumoro et al., 2008).

2.1.1 Starch

Starch is a water insoluble granule that compose the major reserve of polysaccharide in higher plants (Dumitriu, 2005). Starch comprises of two essential polysaccharides, amylopectin and amylose. Both polysaccharides are formed based on chains of 1→4 linked α-D-glucose where amylopectin is extremely branched consisting on average one branch point which is 1→4→6 linked for every 20-25 straight chain remnants, while amylose is significantly linear (Figure 2.1) (Dumitriu, 2005).

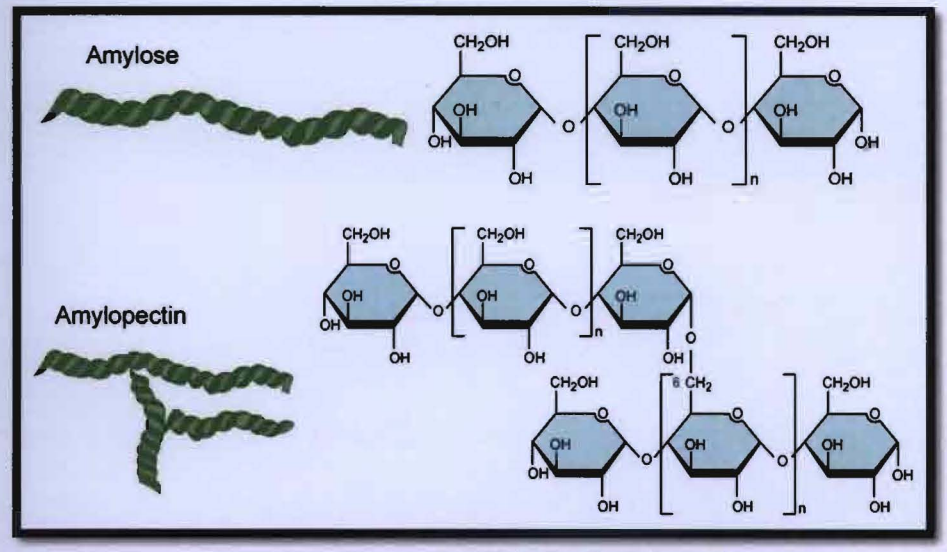

Figure 2.1: Structure of amylose and amylopectin
(Source: https://online.science.psu.edu/chem005_wd/node/7882)

Fibrous sago waste that contains starch can be hydrolysed into valuable glucose to be used as low budget source of nutrient in fermentation procedures for the industry of biotechnology (Kumoro et al., 2008). Starch in sago hampas was constrained by the physical and structural characteristics of lignocellulosic materials. Cell walls in plant cell comprise of microstructural cellulose implanted in a protein and polysaccharide matrix, enclosed by an outer coat of pectin material whereby the starch granules inside this matrix of complex polymer are tough to release (Awg-Adeni et al., 2013).

2.1.2 Cellulose

Cellulose is found in an organised fibrous structure where it is the main component of plant cell wall which confers the structural support for the cell (Agbor et al., 2011). β-(1,4)-glycosidic bonds link the D-glucose subunits to each other and thus making up the linear polymer while the long-chain cellulose polymers are joined together by van der Waals and

hydrogen bonds. This causes the cellulose to be packed into microfibrils which is covered by hemicelluloses and lignin (Kumar et al., 2009).

2.1.3 Hemicellulose

Hemicellulose is a branched, heterogeneous polymer of hexoses (galactose, glucose, mannose), pentoses (arabinose, xylose), and acetylated sugars (Agbor et al., 2011). The branches consist of short lateral chains which is easily hydrolysed and they have a lower molecular weight when compared to cellulose (Agbor et al., 2011). The backbone of hemicellulose is either a heteropolymer or a homopolymer where the short branches are connected by β-(1, 4)-glycosidic bonds and sometimes β-(1, 3)-glycosidic bonds (Kumar et al., 2009).

2.1.4 Lignin

Lignin is a multiplex, large molecular construction comprising of cross-linked polymers of phenolic monomers which is available in primary cell wall, conferring structural support, resistance against microbial attack, and impermeability (Kumar et al., 2009). It is insoluble in water and due to its close relationship with cellulose microfibrils, lignin has been recognized as a main inhibitor to microbial and enzymatic hydrolysis of lignocellulosic biomass (Agbor et al., 2011).

2.2 Pretreatment of Lignocellulosic Biomass

Pretreatment is the disruption of lignocellulosic biomass structure that is naturally resistant to make volatile intermediates such as fermentable sugars to biological procedures (Yang et al., 2011). In theory, the ideal pretreatment activity generates a disrupted, hydrated substrate that is smoothly hydrolysed but prevents the generation of fermentation inhibitor and sugar deterioration products (Agbor et al., 2011). Pretreatment alter the construction of cellulosic biomass to cause cellulose in the plant fibres to be exposed and more accessible. Pretreatment process can be roughly divided into different categories; physical, physicochemical, chemical, biological, electrical, or a combination of these (Kumar et al., 2009).

2.2.1 Steaming pretreatment

Steam pretreatment or steam explosion is the most comprehensively studied and commonly applied physicochemical method of biomass pretreatment (Agbor et al., 2011). Steam pretreatment is an attractive pretreatment process as it makes limited use of chemicals, requires relatively low levels of energy and, depending on the conditions employed, results in the recovery of most of the original cellulose and hemicellulose-derived carbohydrates in a fermentable form (Chandra et al., 2007). The benefits of steam pretreatment also includes the low energy prerequisite compared to mechanical comminution and no environmental expenses or recycling. The traditional mechanical approaches necessitate 70% more energy than steam pretreatment to attain equivalent size reduction (Sun & Cheng, 2002).

The major chemical and physical changes to lignocellulosic biomass by steaming pretreatment are often attributed to the removal of hemicellulose. This improves the accessibility of the enzymes to the cellulose fibrils (Mosier et al., 2005). In this method, high-pressure saturated steam were used to treat biomass, typically initiated at a temperature of 160-260 °C (corresponding pressure, 0.69-4.83 MPa) for few seconds to several minutes, and then the pressure is suddenly reduced, which makes the constituents experience an explosive decompression (Kumar et al., 2009). The biomass-steam mixture is detained for a duration of time to stimulate hemicellulose hydrolysis, and the procedure is dismissed by an explosive decompression. The procedure causes lignin transformation and hemicellulose degradation owing to high temperature, thus increasing the possibility of cellulose hydrolysis (Kumar et al., 2009).

During steam pretreatment, parts of the hemicellulose hydrolyse and form acids, which could catalyse the further hydrolysis of the hemicellulose. This process, in which the in situ formed acids catalyse the process itself, is called 'auto-cleave' steam pretreatment. The role of the acids, is probably however not to catalyse the solubilisation of the hemicellulose, but to catalyse the hydrolysis of the soluble hemicellulose oligomers (Hendriks & Zeeman, 2009).

2.3 Sugar Production from Hydrolysis

Acids or enzymes can be used to break down the cellulose into its constituent sugars. Enzyme hydrolysis is widely used to break down cellulose and starch into its constituent sugars while acid hydrolysis hydrolyses hemicellulose to xylose and other sugars (Yang et al., 2011). In acid hydrolysis, the acid acts as the catalyst to disrupt the glycosidic bonds of starch to yield

maltotriose, dextrin, glucose and maltose contingent on the relative position of the bond under attack as calculated from the end of the chain. Whereas in the enzymatic hydrolysis, enzymes such as glucoamylase, act as the catalyst to break the starch glycosidic bonds to yield valuable glucose (Kumoro et al., 2008).

2.3.1 Enzymatic Hydrolysis

Enzymatic hydrolysis is a multi-step mixed response in which insoluble cellulose is firstly disrupted via the synergistic action of exoglucanases/cellobiohydrolases and endoglucanases at the solid–liquid interface. This primary reaction is complemented by additional liquid-phase hydrolysis of intermediates that are soluble, that is, cellobiose and short celluloligosaccharides, which are catalytically broken down to yield glucose by the achievement of b-glucosidase (Yang et al., 2011). Amylases and glucoamylases are common enzymes used for enzymatic hydrolysis of starch while cellulase and β-glucosidase are usually used for enzymatic hydrolysis of cellulose (Ramos, 2003). Successful enzymatic hydrolysis of cellulosic remnants has been achieved using extremely specific enzymes, however saccharification rate of raw materials that are untreated are generally less than 10% (Chen, 2014). Therefore, effective enzymatic hydrolysis necessitates some method of pretreatment to expose the structure of lignocellulosics as well as starch to enhance its efficiency and rate of hydrolysis (Ramos, 2003).

2.4 Amylase Enzyme

The amylases enzyme can be widely categorised into two main classes of glucoamylase and alpha-amylase. The enzyme alpha-amylase is an endo-1,4 amylase, which decreases the

chain length generating oligosaccharides, and in separation may decrease chain length gradually down to the dimer maltose, which may then be broken down by other enzymes of typical sugar metabolism (Esser et al., 2004). Alpha-amylase randomly break the 1,4-α-D-glucosidic linkages among nearby glucose units in linear amylose chain (Figure 2.2) (Pandey et al., 2008).

Glucoamylase, which is an exo-1,4 amylase break down glucose monomers from the non-reducing end of the polymer. Amyloglucosidase or glucoamylase hydrolyses single glucose units from the non-reducing ends of amylopectin and amylose in a stepwise method. The glucoamylase are proficient of hydrolysing both α-1,6 and α-1,4 linkages (Pandey et al., 2008). Because it disrupts from the non-reducing ends of oligosaccharide, glucoamylase is rate-dependent on the free ends produced by alpha-amylase. Glucoamylase also has several pullulanase function which is a starch-debranching enzyme which breaks down the α-1,6 linkages of amylopectin (Esser et al., 2004).

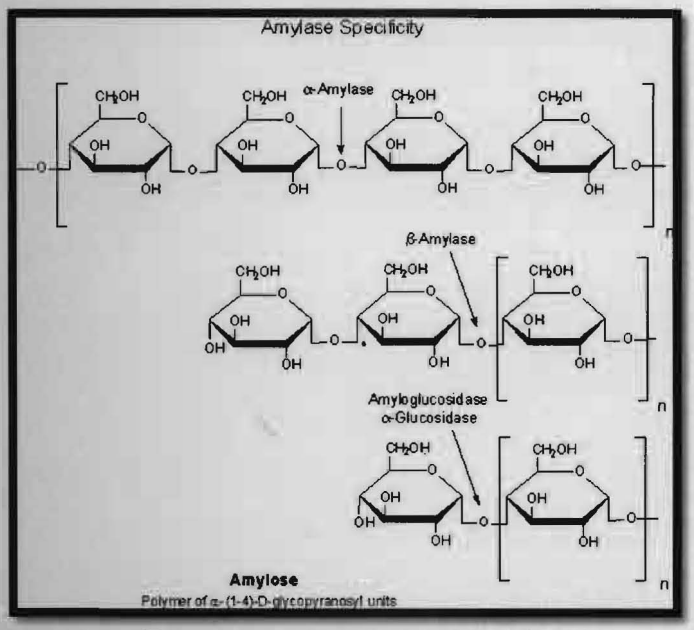

Figure 2.2: Amylase Specificity in cleaving bonds
(Source: http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/learning-center/carbohydrate-analysis.html)

3.0 Materials and Method

3.1 Sampling Preparation

Sago hampas was obtained from Mukah, Sarawak. The sago hampas were dried and ground using techniques adapted from Awg-Adeni et al. (2013). The hampas was packed into porous plastic bags and left to stand for five days under the sun to allow water from the wet hampas to drain off naturally and ensure the sago hampas was completely dried (Figure 3.1). Then, the dried sago hampas was ground using a blender and later sieved to increase the surface area for enzyme accessibility (Figure 3.2, 3.3, 3.4 and 3.5). The sago hampas were then kept in a container.

Figure 3.1: Wet sago hampas left to stand for five days under the sun

Figure 3.2: Sieving activity of dried sago hampas

Figure 3.3: Sago hampas that cannot pass through the sieve

Figure 3.4: Dried sago hampas

Figure 3.5: Blended and sieved sago hampas

3.2 KH_2PO_4 Buffer Solution Preparation

An amount of 1 L of 0.1 M KH_2PO_4 buffer solution was prepared by adding 13.6086 g of KH_2PO_4 to 1 L of distilled water. The buffer solution were then stirred to ensure that the KH_2PO_4 were fully dissolved. The pH of the buffer solution were then analysed using pH meter and later adjusted to pH 5 and pH 4 respectively by adding NaOH or HCl.

3.3 Steaming Pretreatment of Sago hampas

To pretreat the sago hampas, 7% (w/v) substrate loading of sago hampas (7 g) were suspended into 0.5 L Schott bottle that contains 100 ml of KH_2PO_4 (pH 4) buffer solution. This suspension then undergone steaming pretreatment by autoclaving at 121 °C for 20 minutes. After completing the steaming pretreatment, the suspension was cooled down. The same method were applied for 7% substrate load with pH 5 KH_2PO_4 buffer and 10% substrate load with pH 4 and 5 of KH_2PO_4 buffer solution. All experimental runs were done in duplicate.

3.4 Enzymatic Hydrolysis of Starch

3.4.1 Enzymes

The commercial liquefaction enzyme used in this study was Liquozyme SC DS enzyme while the commercial saccharification enzyme used was Spirizyme Fuel enzyme (Novozyme, Denmark). Liquozyme SC DS was an alpha-amylase while Spirizyme Fuel was a glucoamylase.

3.4.2 Enzymatic Hydrolysis

After completing steaming pretreatment, the same sample undergone enzymatic hydrolysis on starch. Firstly, the sample suspension contained in 0.5 L Schott bottle were heated at 85 °C on Cimarec hot plate for 15 min for gelatinisation. After that, 14 µl (0.02%) (w/w) of Liquozyme SC DS enzyme (alpha-amylase) were pipette into the Schott bottle for liquefaction process and stirred manually for 20 min. An aliquot of 2 ml of sample were pipetted out from the suspension. The suspension were then cooled down to 65 °C before adding 7 µl (0.01%) (w/w) of Spirizyme Fuel (glucoamylase) into the mixture and stirred to ensure the solution mixes well. An aliquot of 2 ml of sample were pipetted out from the suspension as sample for 0 min. Then, the suspension were immediately placed in Orbital Incubator Shaker at 50 °C and 100 rpm for saccharification process. An aliquot of 2 ml of sample were pipetted out from the suspension for every 30 min until 90 min reaction time and were kept into centrifuge tubes. All samples collected were centrifuged at 6500 rpm for 10 minutes and the supernatant were filtered by using 0.45 µm nylon paper. The pellet was oven dried before being observed under SEM.

Control for the experiment were also done where sample suspension does not undergo any steaming pretreatment. The samples undergone enzymatic hydrolysis of starch according to the method mentioned above.

3.6 Flow Chart of Overall Methodology

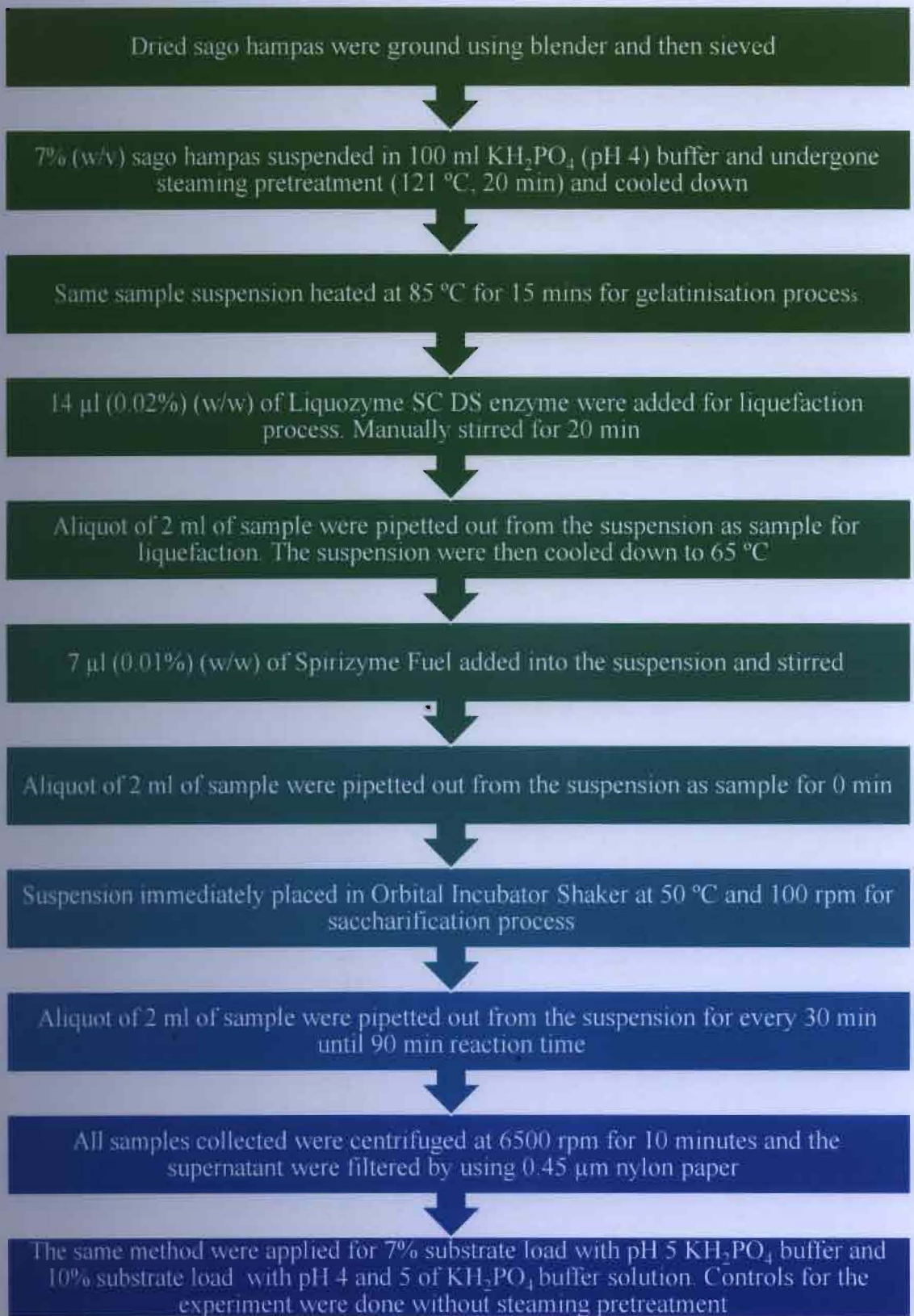

Figure 3.6: Flow chart of overall methodology